Effect of pH on Mycorrhizal Formation Of Slash Pine In Aseptic Culture

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Abstract. Substrate pH strongly influenced mycorrhizal formation and growth of slash pine (*Pinus elliottii* var. *elliottii*) seedlings in aseptic culture. Vermiculite substrates changed the pH of unbuffered nutrient solutions from pH 3.0 to pH 6.4–6.7 in less than 48 hours after autoclaving. Sand substrates caused only slight changes. Phosphate and phthalate buffers were either inhibitory or lethal to *Cenococcum graniforme*, *Laccaria laccata*, and *Suillus luteus* grown in agar medium or aseptic sand culture in combination with slash pine seedlings or alone. Small quantities of peat moss mixed with vermiculite adequately offset the buffering action of vermiculite and established stable pH reactions. Mycorrhizal synthesis occurred with each of the three fungi at pH 4.0, 4.6, 5.6, and 6.6 when the peat moss-vermiculite mixtures were used. All mycorrhizae developed best in less acid substrates. Control seedlings lacking mycorrhizae were significantly less vigorous than those with mycorrhizae. *C. graniforme*, which formed the least mycorrhizae, contributed strongly to seedling growth, especially at pH 4.0 and 4.6. *S. luteus* also formed few mycorrhizae but contributed significantly to seedling vigor in the acid substrates. *L. laccata* formed abundant mycorrhizae and enhanced seedling growth at all pH levels.

MANY FACTORS may influence the distribution and growth of mycorrhizal fungi in forest soils. It has been suggested that pH of the soil influences the distribution of different tree root-fungal associates and consequently, the formation of different mycorrhizal associations. Melin (1953) asserted that certain tree mycorrhizae found in neutral or slightly acid soils may contain fungal symbionts different from those found in acid soils.

Most mycorrhizal fungi of trees are considered acidophilic (Hacskaylo 1957). Tesic (1958) stated that mycorrhizal fungi grow best in acid soils (pH 4 to 5) and that some will form fruiting bodies only in acid media. How (1941), Modess (1941), Mikola (1948), Norkrans (1949), and others have demonstrated the acidophilic nature of various mycorrhizal fungi in synthetic culture media.

The influence of pH on mycorrhizal development in the field and in the greenhouse has received attention. Goss (1960) studied the development of mycorrhizae on ponderosa pine (*Pinus ponderosa* Laws.) seedlings in grassland soils ranging in pH from 5.8 to 6.8, and found mycorrhizae in nursery soils having a pH of 7.8. Björkman (1940) found that soil reactions ranging from pH 4.5 to 5.7 had no apparent effect on mycorrhizal development of pine and spruce seedlings. Richards (1961) observed that mycorrhizae of *Pinus taeda* L. and *P. caribea* Mor. were not inhibited in neutral or slightly alkaline soils, provided the soil nitrate level was low. In these and similar studies many mycorrhizal fungi were probably present in the test soils. Each fungus could

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possibly react differently at each pH level. Information concerning the pH optimum for maximum symbiosis between specific mycorrhizal fungi and tree roots is limited. Shemakhanova (1960) found that *Boletus luteus* (Linn.) Fr. [Now designated *Suillus luteus* (L. ex Fr.) S. F. Gray] formed more mycorrhizae on roots of coniferous seedlings at pH 5.4 than at pH 4.8 or 4.1. We are not aware of any other reports concerning the pH requirements for specific mycorrhizal associations under pure culture conditions.

The purpose of this study was to investigate the influence of pH on the development of slash pine (*P. elliottii* var. *elliottii*) mycorrhizae formed by *Suillus luteus*, *Cenococcum graniforme* (Sow.) Ferd. and Winge, and *Clitocybe laccata* (Scop.) Quel. [Now designated *Laccaria laccata* (Scop. ex Fr.) Berk. and Br.] under Melin's (1921) aseptic culture conditions. These fungi have been reported as symbionts on roots of slash pine (Bryan and Zak 1961, 1962; Zak and Bryan 1963).

Preliminary Investigations

Preliminary experiments were conducted on the activity and stability of various substrates and buffer systems to select a combination suitable for the study. Several researchers have demonstrated the physiological activity of certain organic and inorganic buffer systems, and others have shown that certain substrates can alter pH reactions.

Vermiculite and sand substrates. Vermiculite (Terra-lite) is the substrate presently used in Hacskaylo's (1953) modification of Melin's synthetic culture test for pine mycorrhizae. Vermiculite was substituted for sand to increase aeration and water-holding capacity. Hacskaylo found that vermiculite moistened with nutrient solution would tie up hydrogen ions during autoclaving causing a shift in pH from 4.0 before to 5.4 after autoclaving. Hatch (1937) stated that certain types of sand moistened with nutrients would change from a pH of 6.8 before to 4.2 after autoclaving. The following test was designed to determine the pH stability of Melin's nutrient solution (1921), as modified by Norkrans (1949), in sterile vermiculite[1] and sand[2] substrates.

Screened vermiculite or washed sand was moistened with 25 ml of Melin-Norkrans nutrient, (2:1 v/v), adjusted with 5 percent HCl to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and unadjusted, pH 6.65, in triplicate and autoclaved at 121° C. The nutrient solution, alone, was used as the control. Measurements were made with a Beckman combination pH electrode.

Vermiculite buffered the supplemented nutrient to pH 6.4-6.7 in less than 48 hours, regardless of the original pH (Fig. 1); whereas the sand and control treatments remained reasonably stable. Similarly, Chilvers (1962) reported that vermiculite brought about a rapid rise in soil pH because of its high cation-exchange capacity. This shift in substrate pH toward neutrality may explain why certain hymenomycetous fungi, commonly associated with acid forest soils and mycorrhizae, failed to form mycorrhizae in the vermiculite substrate synthetic culture test. The sand used in this test had a low water-holding capacity and was eliminated as a substrate for mycorrhizal synthesis.

Phthalate and phosphate buffers. Potassium acid phthalate, a widely used buffer (Gortner 1947), neutralized the buffering action of vermiculite when added to Melin-Norkrans nutrient solution. A concentration of 0.02M necessary to stabilize pH at 4.2, however, inhibited growth of *Laccaria laccata* and *Cenococcum graniforme*, and proved lethal to *Suillus luteus*. Bateman (1962) reported that a

[1] Grade 2. Zonolite Brand Vermiculite, 135 LaSalle Street, Chicago 3, Ill.

[2] Grade 2QROK. Pennsylvania Glass Sand Corporation, Two Gateway Center, Pittsburgh, Pa.

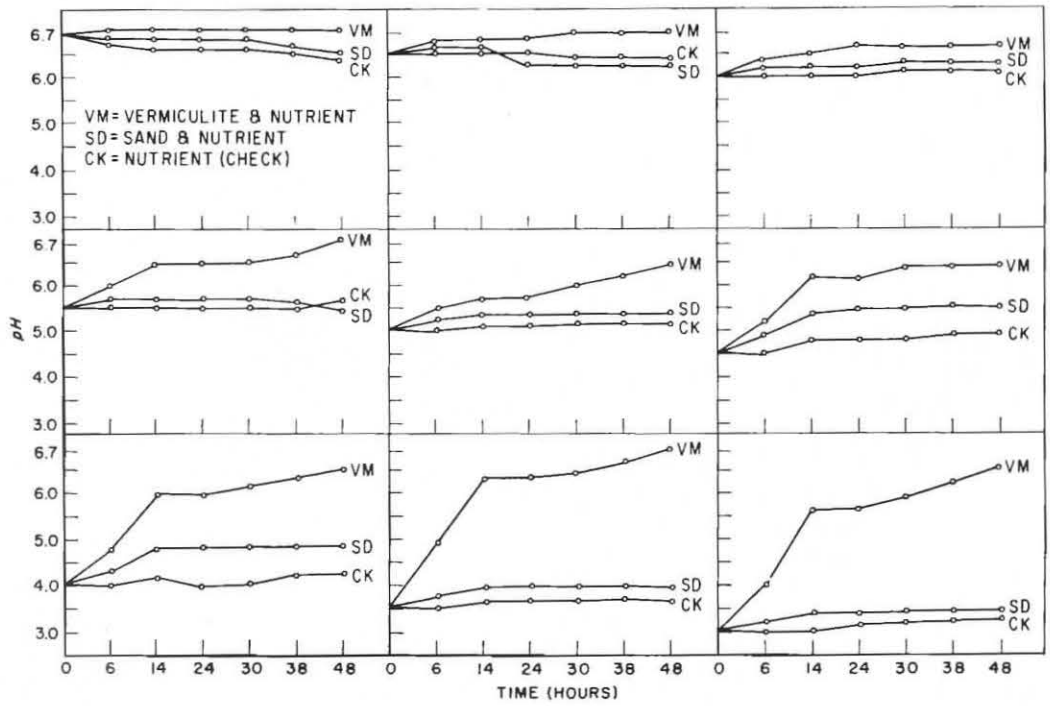

FIGURE 1. *Effect of sterile sand and vermiculite on pH of Melin-Norkrans nutrient solution during a 48-hour period. Initial, adjusted pH indicated on left.*

citrate buffer was toxic to *Thielaviopsis* spp. at high concentrations and was utilized as an additional carbon source at low concentrations. Thimann and Bonner (1948) examined the physiological role of certain buffers and found that citric acid stimulated auxin-induced growth in plants.

Phosphate buffers, prepared according to Gortner (1947), were incorporated in Melin-Norkrans nutrient solution in sterile sand to produce reactions of pH 4.2 and 5.4. All tests were replicated three times. Each pH reaction with test fungi and with and without 3-month old aseptic slash pine seedlings changed after 25 days. Those initially buffered at pH 4.2 varied between pH 4.6 and 5.6, while those buffered at pH 5.4 varied from pH 4.7 to 5.7. Apparently, either secretions by the test organisms or metabolic utilization of the phosphate buffer, or both, brought about the pH changes. Nitsch and Nitsch (1956) demonstrated that certain phosphate buffers were active in the Avena first internode test. Mikola (1948) reported that *Cenococcum graniforme* induced pH changes in phosphate-buffered nutrient solution, indicating the possible metabolic utilization of the phosphates.

These preliminary investigations proved that synthetic buffers have definite limitations when used to stabilize pH for prolonged test periods in complex biological systems.

Peat moss and vermiculite mixtures. Peat moss has been widely used in agriculture as a soil amendment. Hitchcock (1928), Long (1933), and Feustel (1939) incorporated peat in sand cultures to produce a favorable pH, aeration, and water-holding properties for the rooting of many plant species. Peat moss[3] has a strong

[3] Information obtained by personal communications in 1962 from M. T. Graham, Vice-President, Premier Peat Moss Corporation, 535 Fifth Avenue, New York 17, N. Y.

cation-exchange capacity that can alter most alkaline or neutral reactions. The following test was designed to determine the influence of peat moss on the buffering action of vermiculite.

Small quantities of finely ground peat moss were mixed with vermiculite to a volume of 900 ml, moistened with 600 ml of Melin-Norkrans nutrient, and autoclaved. The volumes of peat moss were increased from 20 to 120 ml by 10 ml amounts and each series was replicated three times.

When we determined the pH nine days later we found that peat moss counteracted the buffering action of vermiculite and established stable pH reactions. A ratio of 20 ml of peat moss to 880 ml of vermiculite produced a pH of 6.0; and, a ratio of 120:780, a pH of 4.0. The pH decreased proportionately as the volume of peat moss was increased. These reactions were relatively the same one month later with a variation of ± 0.3 within and between each flask of a treatment.

These substrate mixtures, because of their pH stabilities and comparable aeration and water-holding capacities (variation of 3 ml nutrient/100 ml substrate between ratios 20:880 and 120:780), were selected for studying the effect of pH on slash pine mycorrhizae. It was assumed in this study, as in most other pH studies, that slight variations in nutrient complement can be expected between different pH levels.

Methods of Study

Two-liter Erlenmeyer culture flasks were prepared by adding the following volumes of peat moss and vermiculite: 120 and 780 ml (pH 4.0); 60 and 840 ml (pH 4.6); 30 and 870 ml (pH 5.6); and 0 and 900 ml (pH 6.6). After mixing thoroughly, 600 ml of Melin-Norkrans nutrient solution was added, the flasks plugged with cotton, and autoclaved for 30 minutes at 121° C. Two weeks later two flasks from each mixture were tested but no change from the initial pH was noted.

Medium-size slash pine seeds were selected to minimize initial seedling growth response, as suggested by Righter (1945) and Fowells (1953), surface sterilized with 35 percent hydrogen peroxide (Trappe 1961), and germinated as recommended by Bryan and Zak (1961). Seedlings with radicles from ¾ to 1¼ inches long were planted in pairs about three inches apart in each culture flask.

Five flasks in each mixture were selected at random and inoculated with *Laccaria laccata*, *Cenococcum graniforme*, or *Suillus luteus* grown on Hagem's medium (Modess 1941). Inocula were placed one inch deep and in five locations around the seedlings in each flask. One series of flasks of each mixture remained uninoculated and served as the control. All plantings and inoculations were carried out in an air-filtered transfer room.

The flasks were held in the laboratory under artificial light for ten days to confirm the absence of contamination, then placed in a greenhouse fitted with 30 percent overhead slat shading and their bases immersed in a water bath maintained at 17° C (Fig. 2). Substrate temperature did not exceed 25° C during the test period. The experiment was completely randomized with three mycorrhizal fungi and a control at each of four pH levels replicated five times. The flasks were rerandomized bimonthly on the greenhouse bench.

After four months the flasks were opened and the test fungi reisolated to verify the purity of the cultures. The seedlings were removed and detailed data taken. The total number of mycorrhizae and the total number and length of short and lateral roots were made by individual counts and measurements under a lens-light (10X) apparatus. The number of mycorrhizae or short roots per centimeter of lateral root were calculated from the total length of lateral root measurement obtained from each root system. Dry weights were obtained by drying at 100° C for 24 hours. Analyses of variance were carried out and

FIGURE 2. *Bench layout in greenhouse showing culture flasks immersed in circulating, refrigerated water bath maintained at 17° C.*

significant treatment differences were evaluated according to Duncan's Multiple Range Test (Steel and Torrie 1960) or graphically represented as corrected means.

Representative mycorrhizae or uninfected short roots (control series) were severed and placed in weak chrom-acetic killing solution. These were dehydrated according to Johansen's (1940) tertiary butyl alcohol method, cut 12 microns thick, and differentially stained using Jackson's (1947) safranin-picro-aniline blue method.

Results and Discussion

All seedlings grown with fungal symbionts developed well-formed, ectotrophic mycorrhizae at each pH level. Reactions of the peat moss-vermiculite mixtures at the end of the experiment were within 0.3 pH unit of the initial values.

TABLE 1. Effect of pH, alone, on growth of slash pine seedlings in aseptic culture.

Growth measurement	pH levels in order of rank[1]			
Height, cm	pH 4.0	pH 4.6	pH 5.6	pH 6.6
	19.87	19.25	18.96	17.20
Root wet weight, mg	pH 4.6	pH 4.0	pH 6.6	pH 5.6
	334.91	334.22	317.17	252.54
Total wet weight, mg	pH 4.0	pH 4.6	pH 6.6	pH 5.6
	1195.36	1148.98	1018.86	1014.72
Total oven dry weight, mg	pH 4.6	pH 4.0	pH 5.6	pH 6.6
	242.60	242.35	216.88	201.53

[1] Indicated differences were determined by using Duncan's Multiple Range Test at the 5 percent level. All means underscored by the same line are not significantly different from each other.

Influence of pH on seedling growth. Independent of mycorrhizal fungi, seedlings developed best in substrates adjusted to pH 4.0 and 4.6 and poorest in substrates adjusted to pH 5.6 and 6.6 (Table 1). Significantly better height growth and wet and oven-dry weights were obtained at pH 4.0 and 4.6. Certain root measurements, i.e., number of mycorrhizae or uninfected short roots per cm of lateral root, length of short or lateral roots, and oven-dry weights, were not affected by substrate pH. However, variation in root succulence was indicated since the root wet weights were significant whereas the oven-dry weights were not.

Influence of mycorrhizal fungi on seedling growth. All the mycorrhizal seedling groups, independent of substrate pH, were taller, with more and longer lateral roots, and heavier in all weight measurements than the controls (Table 2). The control seedlings grown free of mycorrhizal fungi had longer short roots than did the mycorrhizal seedlings. This suggests that uninfected short roots growing adjacent to mycorrhizae may be inhibited in their elongation, possibly because of exudates of the free-living fungus mycelia in the substrate or from inhibiting substances translocated from mycorrhizae to the uninfected short roots.

Cenococcum graniforme strongly influenced seedling growth by stimulating an increase in the number of short and lateral roots. The short roots were prima-

TABLE 2. Effect of mycorrhizal fungi on growth of slash pine seedlings in aseptic culture, independent of pH.

Growth measurement	Fungal or control groups in order of rank[1]			
Mycorrhizae per cm[2] of lateral root	*L. laccata* 3.43	*S. luteus* 0.60	*C. graniforme* 0.21	
Height, cm	*L. laccata* 19.84	*C. graniforme* 19.75	*S. luteus* 19.51	Control 16.18
Short roots per cm of lateral root	*C. graniforme* 5.06	*Control* 4.71	*S. luteus* 3.45	*L. laccata* 1.18
Length of short roots, mm	Control 1.65	*S. luteus* 1.41	*C. graniforme* 1.33	*L. laccata* 1.29
Total length of lateral roots, cm	*C. graniforme* 36.75	*S. luteus* 28.25	*L. laccata* 22.96	Control 18.96
Root wet weight, mg	*L laccata* 334.45	*C. graniforme* 329.02	*S. luteus* 332.35	Control 252.02
Total wet weight, mg	*C. graniforme* 1230.81	*L. laccata* 1210.78	*S. luteus* 1083.10	Control 853.23
Root oven-dry weight, mg	*L. laccata* 26.85	*C. graniforme* 25.95	*S. luteus* 21.59	Control 15.05
Total oven-dry weight, mm	*C. graniforme* 267.72	*L. laccata* 246.83	*S. luteus* 220.08	Control 168.73

[1] Indicated differences were determined by using Duncan's Multiple Range Test at the 5 percent level. All means underscored by the same line are not significantly different from each other.

[2] No control series possible for this growth measurement.

rily simple with only a few dichotomously branched, thus corroborating the report by Slankis (1948) that this symbiont does not induce intensive dichotomy in pine roots. *C. graniforme* produced the least number of mycorrhizae yet contributed as much to the various weight measurements of the seedlings as did *Laccaria laccata*, which formed the most abundant mycorrhizae. Possibly these few mycorrhizae were either more effective in absorption than those of *L. laccata* or the absorption by the root system was so increased by the greater number of short roots that it equalled that of seedlings grown with *L. laccata*. Mycorrhizae formed by *C. graniforme* were similar to those described by Mikola (1948) on pine roots. This is the first report of *C. graniforme* forming mycorrhizae in aseptic culture with a southern pine.

Laccaria laccata produced a significantly greater number of mycorrhizae per unit length of root than the other fungi, independent of pH reaction, resulting in an excellent seedling growth response. They were morphologically and anatomically similar to those reported on slash pine by Bryan and Zak (1961). Short and lateral roots of seedlings grown with this symbiont were greatly reduced in both number and length which limited the ramification of the root system. Although few and small in size, the roots were heavier in both wet and dry weights than those of the other seedling groups. These findings suggest that abundant mycorrhizae on a small root system may be as active in absorption as a larger and more ramifying root development.

Seedlings grown with *Suillus luteus* were intermediate in most growth measurements between those of *C. graniforme* and *L. laccata*. This fungus contributed less to the weight of the seedlings than the other fungi, but stimulated more short roots than *L. laccata*.The mycorrhizae formed by *S. luteus* were few in number and similar to those described by Bryan and Zak (1961) on slash pine.

Interaction of mycorrhizal fungi and pH on seedling growth. The number of mycorrhizae per cm of lateral root of seedlings grown with *C. graniforme* remained constant at the different pH levels. However, the effectiveness of this mycorrizal relationship in the different pH

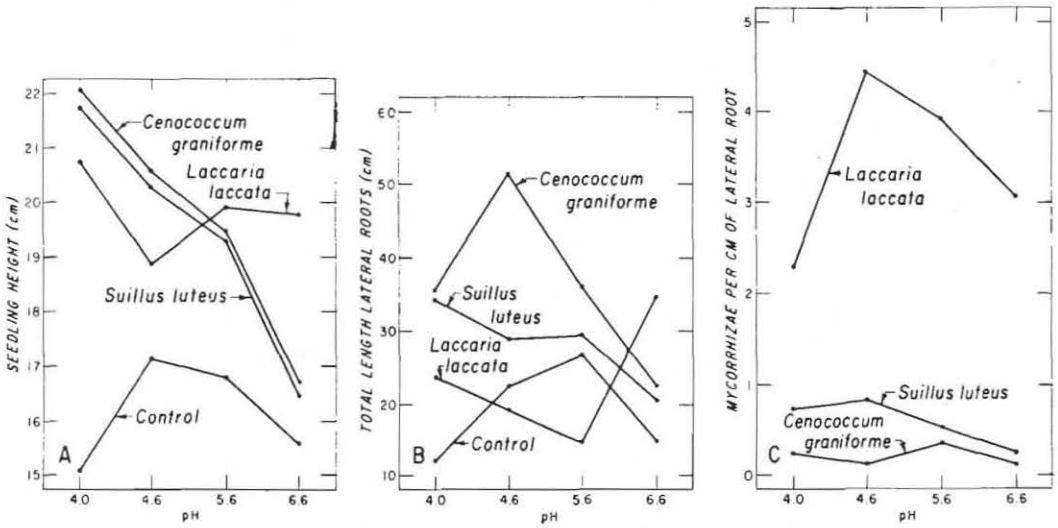

FIGURE 3. *Influence of different pH levels on height (A), total length of lateral roots (B) and mycorrhizae per cm of lateral root (C) of slash pine seedlings grown with and without mycorrhizal fungi in aseptic culture.*

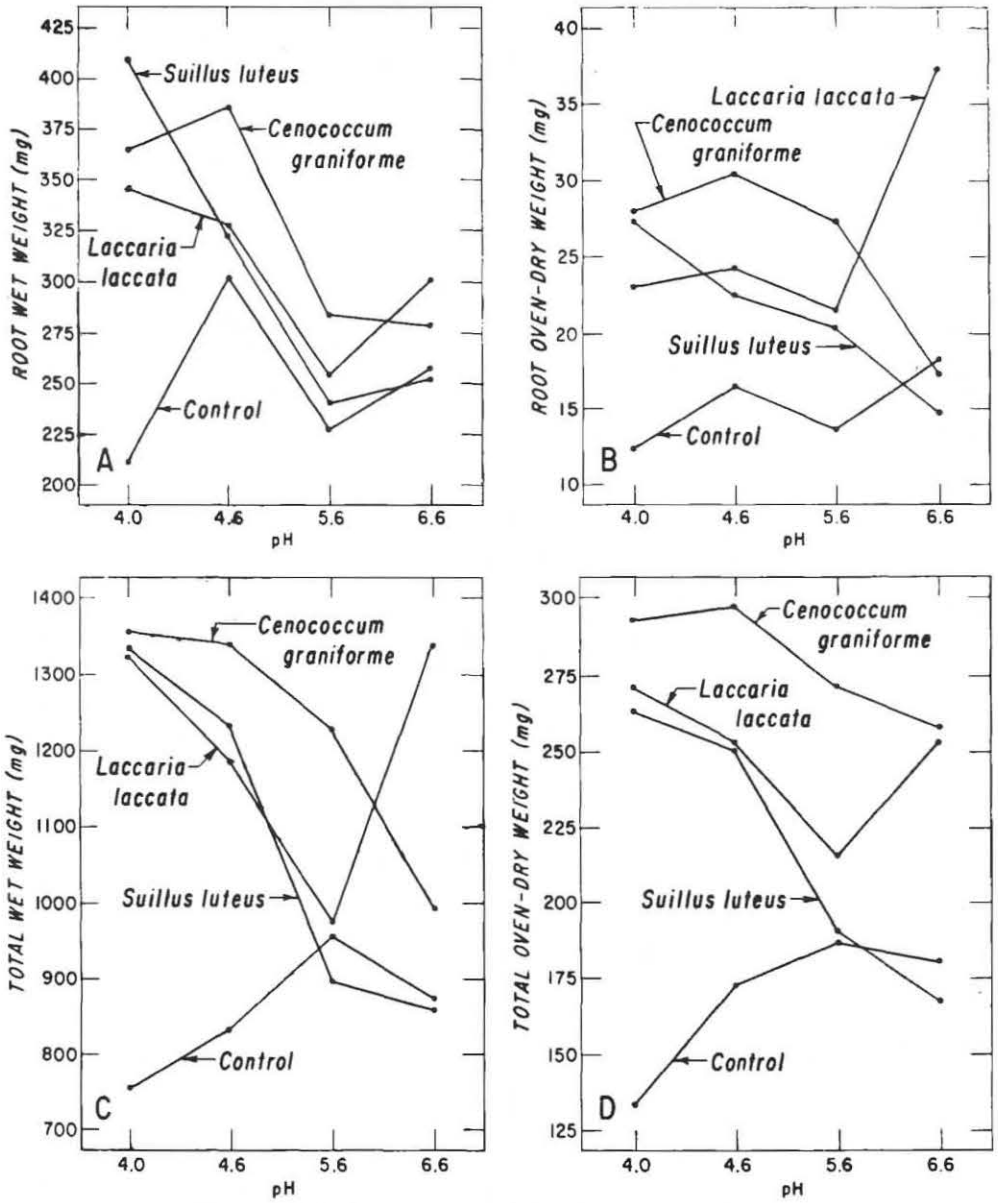

FIGURE 4. *Influence of different pH levels on the root wet (A) and oven-dry weights (B), the total wet (C) and oven-dry weights (D) of slash pine seedlings grown with and without mycorrhizal fungi in aseptic culture.*

reactions was obvious. At pH 4.0 and 4.6 the seedlings were taller, had longer lateral roots, and were much heavier than were those grown at pH 5.6 and 6.6. The seedlings grown with *S. luteus* had only slightly more mycorrhizae at pH 4.0 and 4.6. Here again, possible variable mycorrhizal effectiveness is encountered because seedlings grown with this symbiont developed best in the most acid substrates without having had a marked difference in number of mycorrhizae (Figs. 3 and 4).

Laccaria laccata formed the most

abundant mycorrhizae per cm of lateral root at pH 4.6 and 5.6. Weight and lateral root measurements of these seedlings decreased from pH 4.0 to a low at 5.6, then increased again at pH 6.6. Height growth was somewhat erratic, but appeared to be best at pH 4.0 (Figs. 3 and 4).

The control seedlings were not as vigorous as the mycorrhizal seedlings, especially at the lower pH levels. The roots of these seedlings were not strongly influenced by the pH reactions except the length of lateral roots which was best at pH 5.6 (Figs. 3 and 4). This suggests that the peat moss, which was added in greatest quantity at pH 4.0, had no apparent stimulatory effect, either on root or total seedling growth, for these mycorrhizal-free pine Seedlings.

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